ACTION OF TRITON X-100 ON CHLOROPLAST MEMBRANES

Mechanisms of Structural and Functional Disruption

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ABSTRACT

Addition of Triton X-100 to chloroplast suspensions to a final concentration of 100–200 μM causes an approximate tripling of chloroplast volume and complete inhibition of light-induced conformational changes, light-dependent hydrogen ion transport, and photophosphorylation. Electron microscopic studies show that chloroplasts treated in this manner manifest extensive swelling in the form of vesicles within their inner membrane structure. Triton was adsorbed to chloroplast membranes in a manner suggesting a partition between the membrane phase and the suspending medium, rather than a strong, irreversible binding. This adsorption results in the production of pores through which ions may freely pass, and it is suggested that the inhibition of conformational changes, hydrogen ion transport, and photophosphorylation by Triton is due to an inability of treated chloroplast membranes to maintain a light-dependent pH gradient. The observed swelling is due to water influx in response to a fixed, osmotically active species within the chloroplasts, after ionic equilibrium has occurred. This is supported by the fact that chloroplasts will *shrink* upon Triton addition if a nonpenetrating, osmotically active material such as dextran or polyvinylpyrrolidone is present externally in sufficient concentration (>0.1 mM) to offset the osmotic activity of the internal species.

INTRODUCTION

Triton X-100, digitonin, sodium deoxycholate, and sodium dodecyl sulfate are examples of detergents which have been widely used in solubilizing biological materials and isolating proteins from a lipoprotein environment. Presumably the detergent molecules disrupt gross structure and surround the resulting particles, and thus provide an artificial lipid-like environment which makes soluble the otherwise hydrophobic material. In this type of preparation relatively large amounts of detergents are required. Criddle and Park (1), for instance, used 0.1% (0.03 M) sodium dodecyl sulfate to isolate structural protein from chloroplasts, and Vernon and Shaw (2) employed 3% Triton (0.62 M) to fragment chloroplasts into photochemically active particles.

A more subtle action of detergents which occurs at much lower concentrations is their effect on the structure and permeability of cell membranes. For example, 100 μg/ml saponin lyses red cells (3), and lysolecithin under similar conditions is equally effective at a concentration of 7 μg/ml or about 15 μM (4). In this type of lysis the detergent molecules evidently penetrate the membrane and in some way alter or destroy barriers to ionic movement without disrupting gross membrane struc-

ture. Ions may then freely equilibrate, and water enters in response to fixed, osmotically active materials within; this causes swelling and ultimate lysis. In erythrocytes this is termed "colloid osmotic hemolysis" (5).

This paper will be concerned with the effects of Triton X-100, a nonionic detergent with the structure $CH_3(CH_2)_7C_6H_4(OCH_2CH_2)_{10}OH$.[1] Low concentrations of Triton inhibit photophosphorylation (6), light-dependent hydrogen ion transport (7), and light-induced structural changes in chloroplasts (8). Vernon and Shaw (9) have made a detailed study of uncoupling of photophosphorylation by Triton and found that Triton acts as a true uncoupler at 0.007% (about 100 μM), stimulating ferricyanide photoreduction and completely inhibiting photophosphorylation. Triton also induces extensive swelling, and Neumann and Jagendorf (6) have reported that chloroplasts in Triton solutions swell to three to four times their original volume. However, since the mechanism by which Triton produces these effects is obscure, we have attempted to clarify the manner in which it interacts with chloroplast membranes. The ultrastructural alterations produced by Triton and their relation to the function of chloroplast membranes will also be described.

METHODS

Chloroplast Preparation

Chloroplasts were isolated by homogenizing commercially available spinach, with midribs removed, in 0.35 M sodium chloride buffered with 0.04 M Tris-HCl at pH 8.0. The homogenate was filtered through eight layers of cheesecloth and the filtrate was centrifuged for 1 min at 250 *g* to remove cell debris. The supernatant was then centrifuged for 5 min at 750 *g*, and the resulting pellet was washed once by resuspension in fresh isolation medium and centrifuged a second time at 750 *g*. In experiments chloroplasts were added to 0.1 M sodium chloride or 0.1 M sodium acetate to a final concentration of about 15 μg chlorophyll per ml. Chlorophyll was determined by the procedure of MacKinney (10).

Functional Measurements and Volume Changes

Light scattering (90° at 546 mμ), absorbancy (546 mμ), and pH changes induced by illumination were measured as previously described (11, 12). ATP formation was calculated from the rate of pH change 30 sec after chloroplast suspensions were illuminated under phosphorylation conditions (13). Chloroplast volume was estimated with a Coulter counter, with the automatic particle size distribution analyzer and a 50 μ orifice. Volumes given in the Results section, therefore, represent distribution peaks, rather than average volumes.

Electron Microscopy

A 50% glutaraldehyde solution (Fisher Biological Grade) was distilled at 100° and filtered through Norit activated charcoal. This resulted in a slightly alkaline 8% solution, which was added to chloroplast suspensions in the dark to a final concentration of 2% for fixation. After 1 hr the suspension was centrifuged for 10 min at 700 *g* and the resulting pellet stained for 1 hr with 1% buffered osmium tetroxide, followed by acetone dehydration and embedding in Epon. Specimens were sectioned with a Porter-Blum MT-1 microtome. Sections were poststained with Reynold's lead citrate, and then photographed with a Siemens Elmiskop II microscope.

Triton Adsorption

During the course of these experiments it was desired to measure Triton taken up by chloroplasts; therefore, a quantitative determination of Triton concentration at μmolar levels was necessary. Triton solutions in distilled water or ether were found to have ultraviolet absorption peaks at 276 and 225 mμ. The peak at 276 mμ has previously been used to measure Triton concentration (14) but was not intense enough for the highly dilute solutions used in the present experiments. The peak at 225 mμ was quite intense, with an extinction coefficient of about 9650 $M^{-1}cm^{-1}$. This was found to provide a quantitative measure of Triton concentration. However, in 0.1 M salt solutions this peak was broadened and was no longer proportional to concentration. Therefore, the following procedure was evolved: chloroplasts were added to varying concentrations of Triton in 0.1 M sodium acetate solutions and allowed to stand for 5 min at room temperature, then centrifuged at 10,000 *g* for 10 min. One volume of the supernatant was shaken with two volumes of diethyl ether in stoppered tubes, then allowed to clear for 5 min, and the optical density of the ether extract was read at 225 mμ. With this procedure it was possible to determine the amount of Triton which had disappeared from the supernatant, and this was assumed to be taken up by the chloroplasts. In some experiments at higher concentrations of Triton and chloroplasts the treated chloroplasts were resuspended in fresh medium and the amount of Triton released from the chloroplasts after 5 min was directly measured in the same manner. The relative ease with which Triton concentrations can be determined, as described above,

[1] Rohm & Haas Co., Philadelphia, Pa.

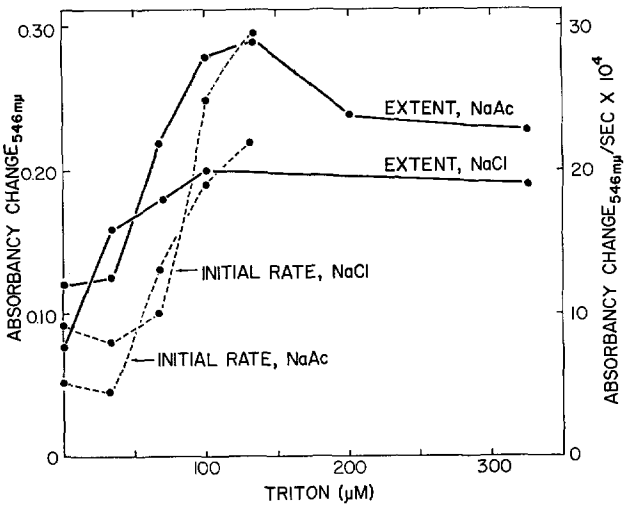


FIGURE 1 Swelling of chloroplasts induced by Triton in the dark. Swelling was measured by the decrease in absorbancy of chloroplast suspensions at pH 6 over a 10 min period. The initial rate and extent of swelling after Triton addition are given as the absorbancy change per second and absolute decrease in 10 min.

readily lends this particular detergent and technique to general studies of detergent effects on biological membranes.

RESULTS

Chloroplast Volume and Functional Parameters

The action of Triton additions in causing swelling and inhibiting various responses of chloroplasts to light has been well documented (6–8). It was of interest to compare the minimal concentrations at which Triton produces these effects, since similar inhibitory concentrations would suggest a similar mode of action. In previous studies (15) it has been shown that the presence of weak-acid anions has a profound effect on the manner in which chloroplasts respond to illumination. Therefore most experiments were performed in both sodium chloride and sodium acetate solutions to provide a correlation with the earlier work.

Chloroplast Swelling Produced by Triton

Chloroplast volume has been found to have an approximately inverse linear relation to the absorbance of chloroplast suspensions (16). Absorbancy of suspensions could, therefore, be used to estimate the extent and rate of swelling induced by varying concentrations of Triton. When sufficient Triton is added to chloroplast suspensions in the dark, a large decrease in absorbancy occurs which proceeds for several minutes and reaches a plateau after about 5 min. Fig. 1 shows both rate and extent of absorbancy decrease at varying Triton concentrations for chloroplasts suspended in 0.1 M sodium acetate or sodium chloride. The absorbancy decrease became maximal between 100 and 150 μM Triton and, although the rate also increased at higher Triton concentrations, above 100 μM the initial kinetics were too rapid to measure with accuracy. As expected with dark-incubated chloroplasts, there was little difference between results with sodium chloride and those with acetate, suggesting that weak-acid anions have little effect on the swelling process in the dark.

These results were confirmed by measuring Coulter counter volume over the same ranges of time and concentration. The volume increased from 30 to 80 μ^3 in 100–200 μM Triton over a period of 10 min; this shows that the absorbancy decrease represented a true increase in volume (Fig. 2). Furthermore, swelling again was not instantaneous. The volume increased throughout the 10 min period until the chloroplasts reached a final volume of 80–90 μ^3.

Inhibition of Light-Induced Conformational Changes

Chloroplasts illuminated in sodium chloride show an increment in 90° light-scattering which is maximal at pH 6 and is accompanied by an *increase* in volume (12, 17). Absorbancy under these conditions *decreases* over a period of several minutes, reflecting the volume increment (18). The presence of weak-acid anions generally enhances

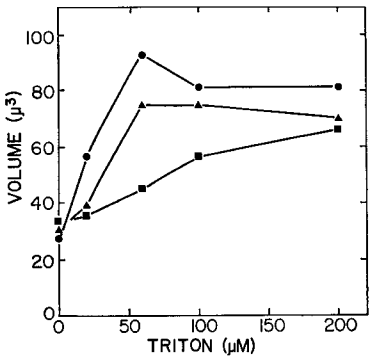


FIGURE 2 Swelling of chloroplasts induced by Triton. Volume increments were measured with a Coulter counter at 2, 5, and 11 min incubation. Since the analyzer requires 2 min to determine the peak volume distribution, these values actually represent averages of time intervals from 1–3, 4–6, and 10–12 min after Triton was added. ■, ▲, ● represent volume increments at 2, 5, and 11 min respectively.

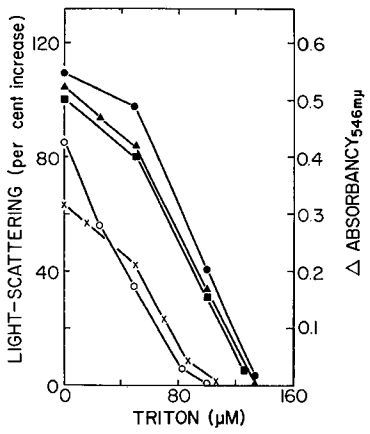


FIGURE 3 Effect of Triton on light-induced conformational changes. Chloroplasts were suspended in 0.1 M NaCl and NaAc at pH 6 with 20 μM phenazine methosulfate (PMS), and illuminated with broad band red light at an intensity of 1,000 footcandles. ■, ▲, ● are light scattering increments by respectively 5, 10, and 20 μg chlorophyll per ml in sodium acetate; ○ shows light-scattering increments by 15 μg chlorophyll per ml in sodium chloride; × shows the absorbancy change in sodium acetate. Suspensions were incubated for 2 min in the dark, illuminated, and maximum responses measured 1–2 min later.

the light-scattering response, and causes a *decrease* in volume. In this case, absorbancy of the suspension *increases*, as would be expected from the volume decrease.[2] All of these responses are inhibited by Triton added before illumination, and are quickly reversed by the addition of Triton during illumination. Fig. 3 shows the effect of varying Triton concentrations on the light-scattering and absorbancy responses of chloroplasts illuminated in chloride or acetate media. Chloroplasts in the acetate medium are somewhat more resistant to Triton, but 100–150 μM Triton concentrations completely inhibit all of the responses. This experiment also shows that doubling or halving the chloroplast concentration does not alter the inhibiting concentration, a point which will be discussed later.

Inhibition of Light-Induced Hydrogen Ion Movements and Photophosphorylation

It has been previously demonstrated that light-induced light-scattering increments and volume changes described above are linked to light-dependent hydrogen ion transport into the chloroplast (12) which can be measured as a pH change in the medium. It has further been suggested (12) that the light-scattering changes in sodium chloride solutions reflect the resulting acidification of the chloroplast interior which would cause a reversible "precipitation" of internal chloroplast proteins. In sodium acetate the result of hydrogen ion transport has been postulated to be an outward transport of acetate (as acetic acid) which results in shrinkage and enhances the light-scattering change.[2] Since Triton strongly inhibits light-induced absorbancy and scattering increments, it would, therefore, also be expected to release the pH gradient; this would result in an observed inhibition or reversal of the pH change at similar concentrations.

Jagendorf and Neumann (7) and Neumann and Jagendorf (6) have indeed shown that addition of Triton inhibits the pH change, or reverses it after it has occurred during illumination. In the present experiments the inhibition of the pH change by varying concentrations of Triton was studied (Fig. 4). The pH change was completely inhibited by 70 μM Triton in sodium acetate and by 130 μM Triton in sodium chloride. It is interesting that the pH change does not simply go to zero as Triton levels are increased, but protons are actually produced and the medium becomes more acidic during illumination. It is probable that this light-

[2] Crofts, A. R., D. W. Deamer, and L. Packer. 1967. *Biochim. Biophys. Acta.* 131:97.

dependent cyclic production of hydrogen ions observed after disruption of chloroplast membranes by Triton may be associated with the reduction and oxidation of the phenazine methosulfate (PMS) present as an electron carrier, and further study of this problem is now in progress.

Photophosphorylation was generally more sensitive to Triton than other parameters of chloroplast function, as seen in Fig. 4. In sodium acetate it was almost totally inhibited by 60–80 μM Triton, and by 100–120 μM Triton in sodium chloride. The lower inhibiting concentration in acetate is probably a function of the mild uncoupling action of this anion (19). The inhibiting concentrations of Triton were in the same range as those necessary for inhibition of the pH change, as previously noted by Neumann and Jagendorf (6), and agree with their result of 0.008% (125 μM) to produce nearly complete inhibition.

Chloroplast Ultrastructure

Since Triton has such a profound effect in causing chloroplasts to swell and in inhibiting their functional capacity, ultrastructural alterations should be readily apparent and perhaps should aid in understanding the basic inhibitory process. We have attempted to correlate structural changes with the functional disruption occurring at three Triton concentrations: 50 μM, at which partial inhibition of function and some swelling has occurred; 100 μM, at which nearly all function is inhibited; and 200 μM, at which the volume increment is maximum.

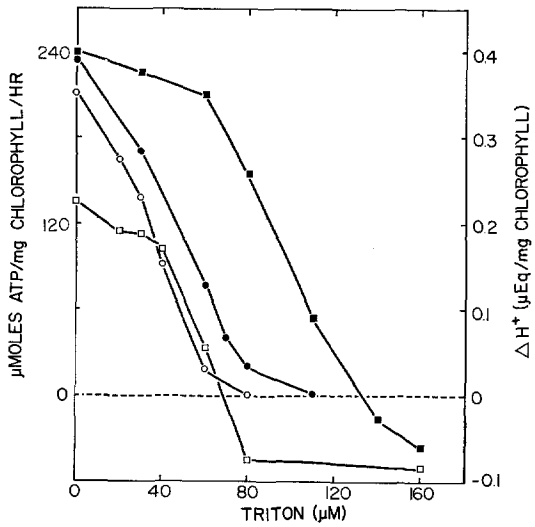


FIGURE 4 Effect of Triton on light-dependent hydrogen ion uptake and phosphorylation. ■ shows pH change (plotted as hydrogen ions taken up per mg chlorophyll) in 0.1 M NaCl, 2 mM K-phosphate, 5 mM $MgCl_2$, and 20 μM PMS at pH 7.3–7.4. ● is photophosphorylation in the same medium, with 0.5 mM ADP added. □ shows pH change, and O, photophosphorylation, in a similar medium under the same conditions as above, except that chloride was replaced by acetate. Chloroplast concentration represents 28 μg chlorophyll/ml.

Control chloroplasts in 0.1 M sodium acetate are shown in Fig. 5. These were, for the most part, Class II chloroplasts (20) that lack outer membranes, although a few outer membranes remained intact. Grana were generally well formed and individual lamellae were readily visible. Swelling, first apparent at 50 μM Triton, was in the form of small vesicles appearing within the internal structure of the chloroplast (Fig. 6). Not all chloroplasts were equally affected, and many seemed normal. In swollen chloroplasts the vesicles were bounded by two separate membranes and seemed to be formed by the successive unfolding of grana lamellae.

At 100 μM Triton, where most function had been lost, chloroplasts were all in the swollen state (Fig. 7). Grana membranes were beginning to be drawn out into long strands and the normal lamellar structure was lost. At 200 μM Triton (Fig. 8) the chloroplasts were swollen even further. It can be seen that each chloroplast is enclosed in a balloon-like membrane, with hundreds of smaller vesicles enclosed within. These vesicles appear to be bounded by only single membranes. Grana membranes have formed long strands which typically end in a closed loop formation.

The ultrastructural changes described above are consistent with the results from absorbancy and Coulter counter studies which indicated a general swelling process induced by Triton. "Lysis" occurred at 0.5–1.0 mM Triton. This probably represented the bursting of the balloon-like enclosing membranes and the release of the inner vesicles. These could no longer be centrifuged at 10,000 *g* to form a pellet after fixation and were not studied.

Interaction of Triton with Membranes

There are several ways in which Triton may interact with chloroplast membranes: Triton molecules may bind to the membrane strongly and irreversibly, so that an absolute amount of Triton

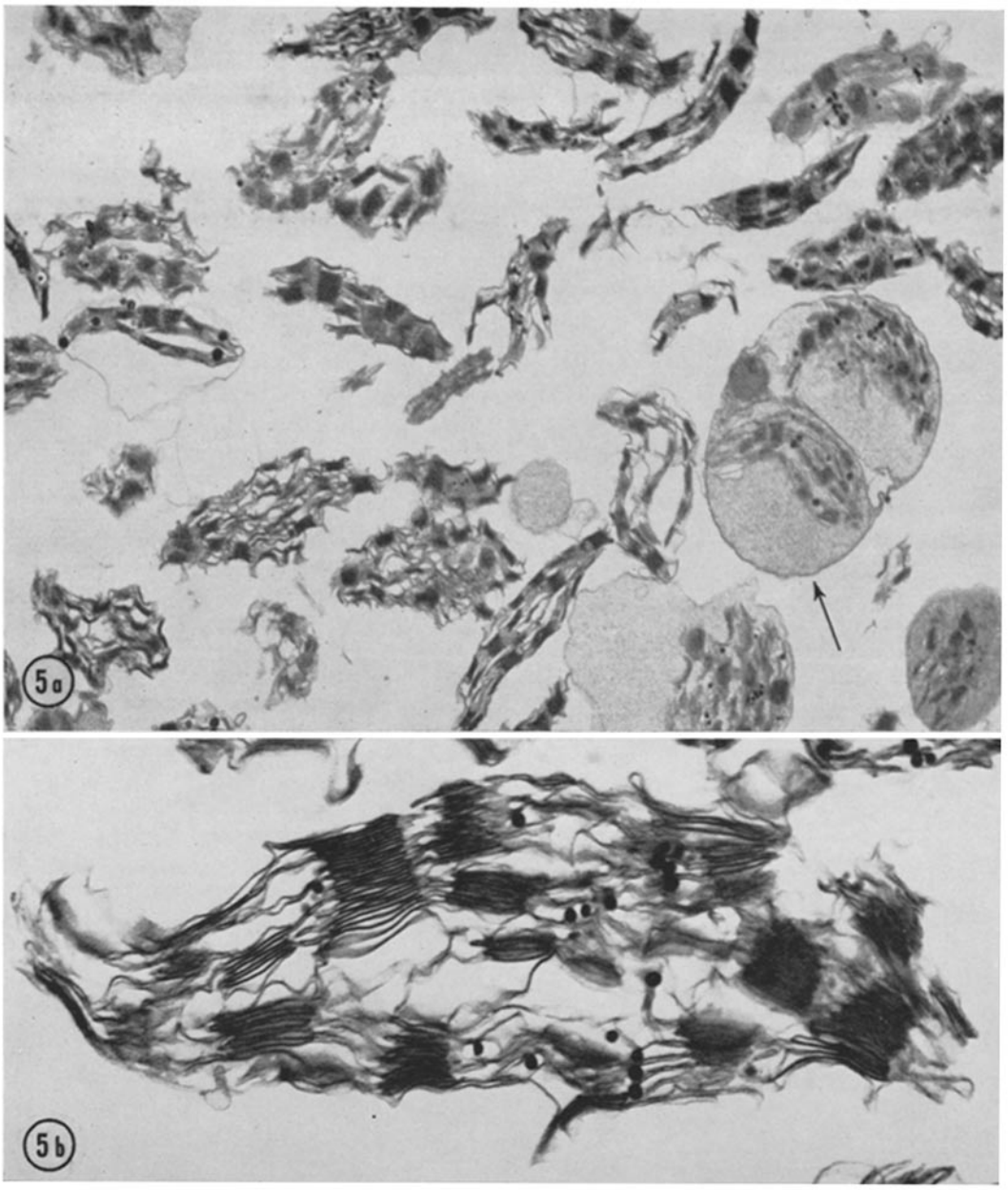


FIGURE 5 Control chloroplasts in 0.1 M NaAc, pH 6.0. (*a*) Low magnification view. Most chloroplasts lost their outer membranes, but a few remained intact (arrow). × 6000. (*b*) Higher magnification view. × 20,000.

is necessary to swell and disrupt a certain number of chloroplasts. An alternative is that Triton may equilibrate between the medium and membrane phases, so that a certain concentration of Triton must be present to have an effect.

A choice between these two possibilities could be made by varying the chloroplast concentration while keeping Triton concentration constant during measurement of any of the physiological parameters. If Triton is binding irreversibly to the

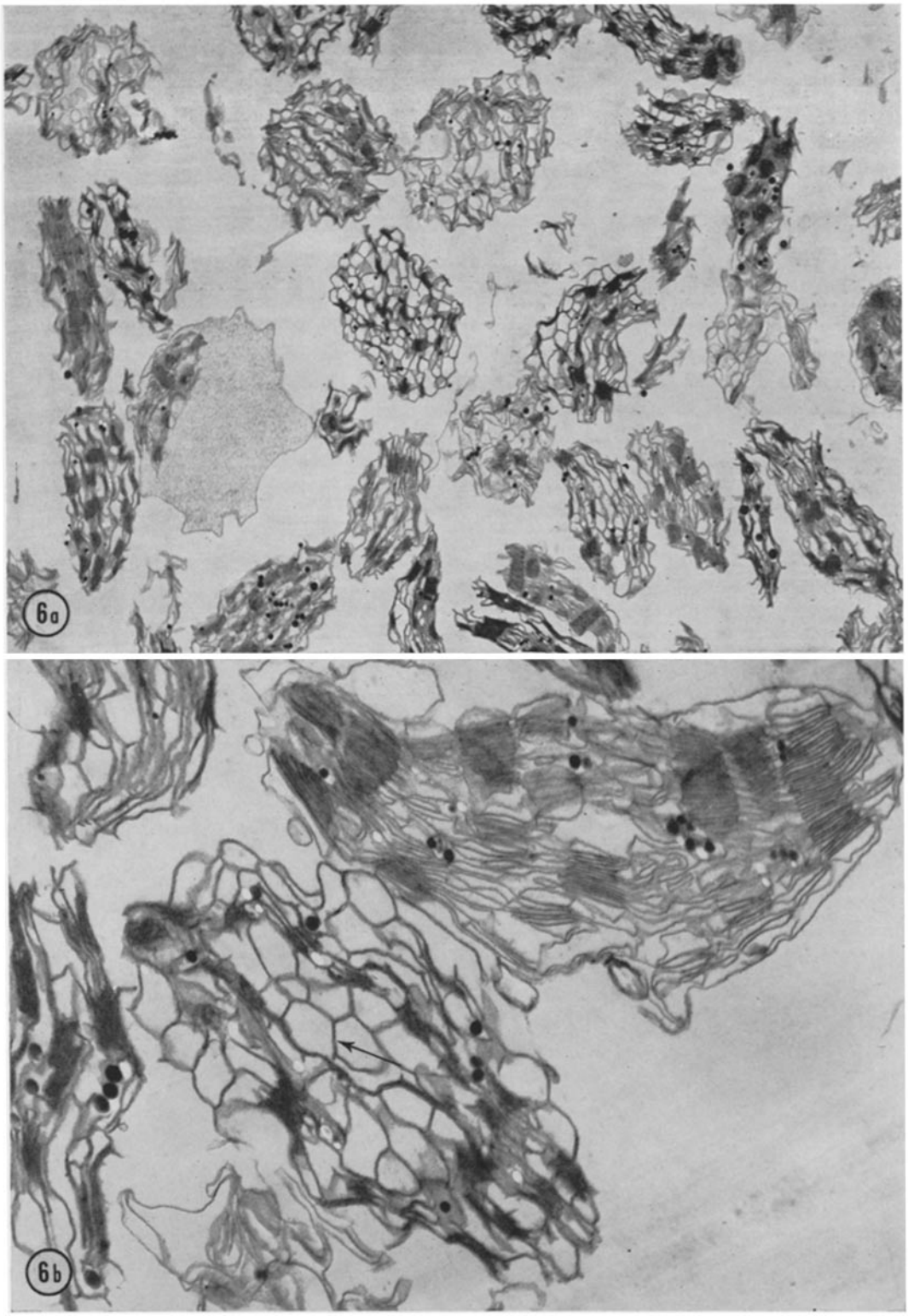


FIGURE 6. Chloroplasts in 0.1 M NaAc, pH 6.0, 50 μM Triton. Suspensions were allowed to incubate for 2 min before fixation with 2% glutaraldehyde. (*a*) × 6000. (*b*) Higher magnification view showing vesicle formation (arrow). Dual membrane structure of vesicle walls is readily apparent. × 20,000.

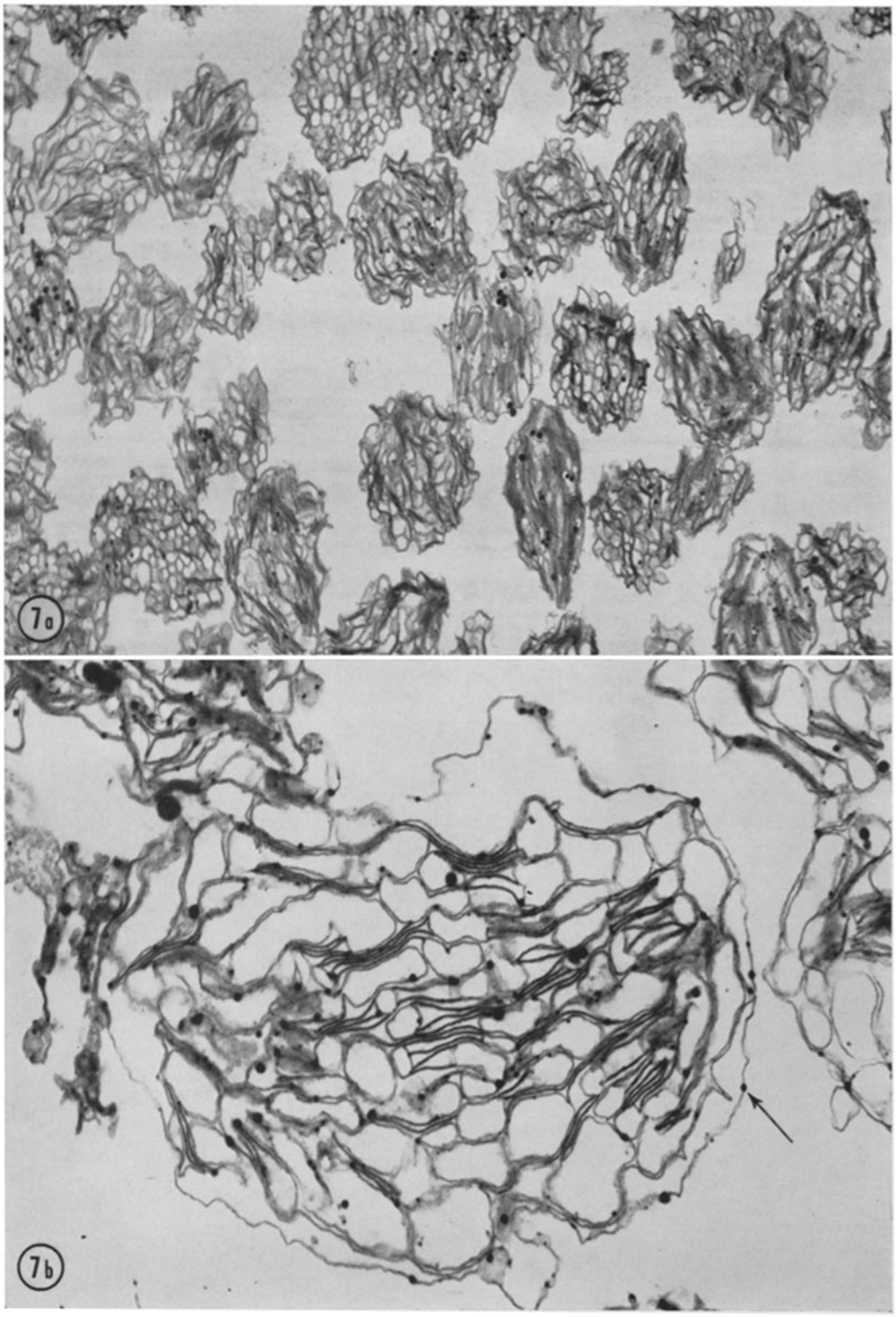


FIGURE 7 Chloroplasts in 0.1 M NaAc, pH 6.0, 100 μM Triton. (*a*) At this concentration all chloroplasts are in the swollen state. × 6000. (*b*) An enclosing membrane with attached osmiophilic granules has formed around the swollen chloroplast (arrow). × 20,000.

membrane, then doubling the chloroplast concentration should reduce the effective amount of Triton present and the measured inhibition should correspond to the lower effective level. On the other hand, if the effect involves a partition between the medium and membranes, doubling the chloroplast concentration should have little or no effect.

The latter seems to be the case, as seen in Fig. 3. Increasing the chloroplast concentration from 5 to 20 μg chlorophyll per ml only slightly changed the inhibiting concentrations of Triton. It can be concluded that the Triton effect is mainly dependent on its partition between the suspending medium and chloroplast membranes.

This conclusion is supported by an experiment in which Triton adsorption by varying amounts of chloroplasts was measured, as described in Methods. These results are shown in Fig. 9 and Table I. The amount of Triton taken up was roughly proportional to chloroplast concentration, but only a fraction of the Triton present was adsorbed; this confirmed that an equilibrium, rather than strong, irreversible binding, existed. If Triton was strongly bound to chloroplast membranes, most of the Triton present in solution would be expected to come down with the chloroplasts, rather than only a small fraction.

Vernon and Shaw (9) noted that they had found it impossible to readily remove Triton from treated chloroplasts and suggested that a tight complex may be forming. However, under our conditions reversible binding could be directly measured by simply resuspending treated chloroplasts in fresh media. This method of determining Triton uptake was not satisfactory in lower concentration ranges since the very small amount absorbed and then released by washing could not be measured accurately. Furthermore, since a partition would still exist during the washing procedure, not all of the Triton could be expected to go back into solution. Therefore, we chose to measure disappearance of Triton from the medium for the quantitative experiments reported above. However, in higher concentration ranges of Triton and chloroplasts it was possible to measure release of Triton accurately, and these results also indicated a highly reversible adsorption. For instance, chloroplasts (35 μg chlorophyll per ml) suspended in 10 ml of 200 μM Triton (in 0.1 M sodium acetate, 10 mM Tris, pH 8) took up 1.6 μmoles Triton per mg chlorophyll as measured by disappearance of Triton from the medium. When the treated chloroplasts were resuspended in 10 ml fresh medium, 1.0 μmole Triton per mg chlorophyll was released. This is what would be expected, considering that a partition between membrane and aqueous phases would still exist during washing. That the ultraviolet absorption spectra of the ether extracts in all experiments could not be distinguished from those of known Triton samples indicated that no interfering substances were being released from the chloroplasts by Triton treatment.

It is interesting to calculate the amount of Triton adsorbed in terms of the area occupied by Triton on the membrane surface of the chloroplast. If one assumes a total membrane area of 800 μ^2 per chloroplast (21), 40 A^2 for the cross-sectional area of a triton molecule,[3] and 10^9 chloroplasts per mg chlorophyll,[4] the values shown in Table I can easily be calculated. These indicate that, at concentrations which inhibit function and alter structure, enough Triton is bound to chloroplasts to cover approximately one-tenth to one-half of their membrane surface area.

There are also two alternative ways in which Triton adsorption to chloroplast membranes can cause swelling: (*a*) Triton molecules, by physically occupying space within the membranes, may be expanding the total area of the membranes by a process similar to that described by Schulman and Rideal (22) when surface active molecules penetrate and expand monolayers after injection beneath the interface; (*b*) the second possibility is that the swelling is entirely osmotic: the Triton molecules may produce pores of some sort which allow passage of ions into the chloroplast interior, and result in a situation similar to "colloid osmotic hemolysis" as observed in erythrocytes which have been treated with surface active agents (5).

It is not possible to rule out some contribution by penetration and expansion, and in fact a certain amount of this would be expected, since Triton definitely is taken up by chloroplasts and must penetrate the membranes. However, the main effect is due to osmotic swelling, as demonstrated by the following evidence.

The adsorption of Triton to membranes occurs very rapidly, since ion gradients are released within seconds. However, the actual swelling

[3] Deamer, D. W. Unpublished results. Estimated from area occupied by a Triton monolayer.

[4] Figure taken from Coulter counter studies.

process as measured by absorbancy decrease and the Coulter counter takes place over a period of several minutes. If swelling was mainly due to penetration and expansion of membranes by Triton, one also might expect it to occur very rapidly.

In an attempt to find a more critical test of this point, small ions in the chloroplast suspensions were gradually replaced by nonpenetrating, osmotically active materials such as dextran, polyvinylpyrrolidone (PVP), and bovine serum albumin (BSA). If swelling was mainly due to penetration and expansion, it should occur equally well in high concentrations of nonpenetrating molecules. Surprisingly, not only was there no swelling upon addition of Triton to this system, but the chloroplasts decreased in volume, as indicated by a rapid increase in absorbancy. Since control experiments showed that absorbancy increments occurred only if chloroplasts were present, no aggregate was forming between Triton and the additives. It was possible that aggregation between chloroplasts might account for the absorbancy increase, but Coulter counter volume studies showed a true decrease in volume without loss of particle number. Finally, electron micrographs clearly showed a typical shrinkage, with grana lamellae tightly packed together (Fig. 10).

Certainly in this case penetration of membranes by Triton molecules could not be expanding the chloroplasts. However, the shrinkage could be explained in terms of pore production and ionic equilibration followed by movement of water outward to an apparently hypertonic medium. If this reasoning is correct, the amount of shrinkage in chloroplasts treated with Triton in the presence of added dextran or PVP should be controlled by the external concentrations of the additives. Fig. 11 shows the absorbancy change plotted against dextran and PVP concentration. Shrinkage occurs after Triton addition only when dextran is present in concentrations greater than 1.25%. Below this concentration the chloroplasts swell as usual. The osmolarity of dextran (mol wt 200,000–275,000) at this concentration is 0.05 milliosmol. The same experiment with PVP (mol wt 40,000) gave a result of 0.6%, or 0.15 milliosmol.

DISCUSSION

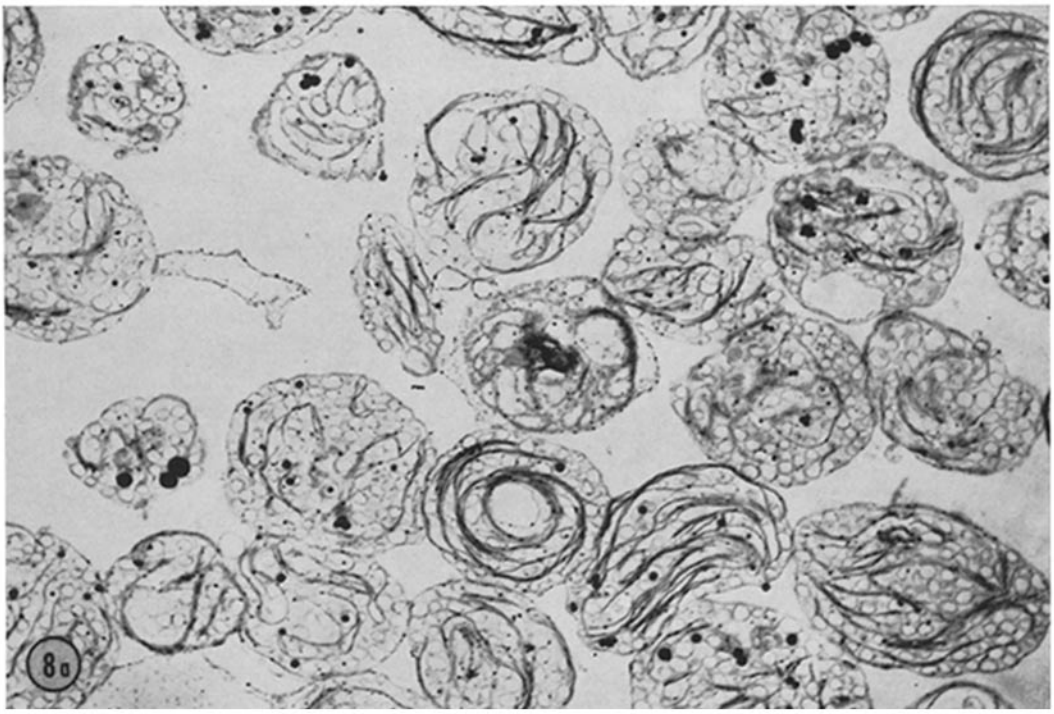
This investigation has established that Triton is taken up by chloroplasts in a manner which involves a partition between the medium and chloroplast membranes, rather than by a strong, irreversible binding. This uptake causes ultrastructural alterations consisting of a generalized swelling of all membrane structures followed by vesicle formation. In the Triton concentration range of 50–200 μM, swelling initiated by Triton occurs over a period of several minutes. However, the inhibition of several parameters of chloroplast function by Triton occurs within a matter of seconds and at similar concentrations.

In the discussion to follow, it will be argued that the main disruptive effect of low concentrations of Triton is the production of pores in the membrane through which ions, including protons, may pass freely. Since Triton has an immediate effect on chloroplast function, and since Triton-induced swelling occurs over a period of several minutes, the swelling process and consequent structural alterations probably are also results of Triton adsorption and pore formation, rather than being in themselves the prime cause of functional inhibition.

Ultrastructural Basis of Triton Action On Chloroplasts

Detergent effects on chloroplasts have been previously reported by Itoh et al. (23) who used Coulter counter techniques to measure volume changes which occurred during the structural disruption of chloroplasts by dodecyl benzene sulfonate (DBS). Their data and the present results agree reasonably well. Chloroplasts can approximately triple in volume before lysing in either detergent. It is interesting to note that the most

FIGURE 8 Chloroplasts in 0.1 M NaAc, pH 6.0, 200 μM Triton. (*a*) All organized grana structure has disappeared. × 6000. (*b*) Unattached vesicles have appeared whose walls appear to have only a single membrane, and the outer enclosing membrane has swollen to a balloon-like state. Darker membranes which appear to be remnants of grana lamellae typically end in closed loop formations (upper arrow). Most osmiophilic granules are enclosed within vesicles, rather than floating in the space between vesicles (lower arrow). × 20,000.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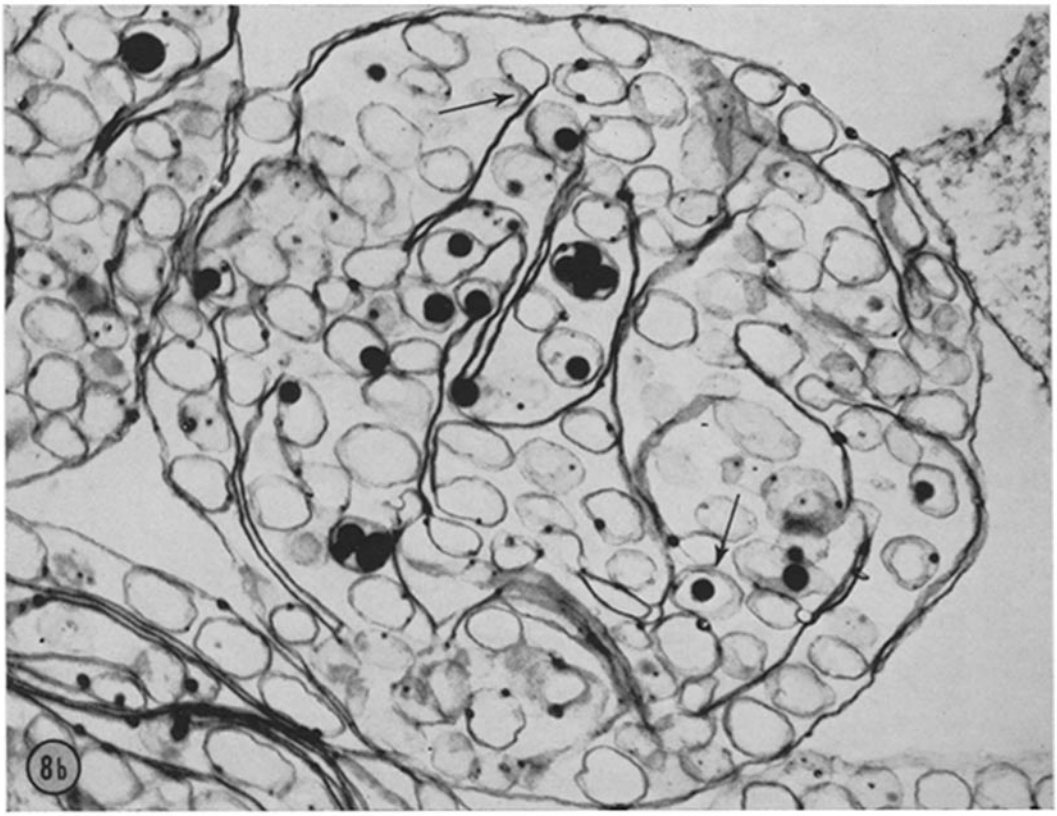
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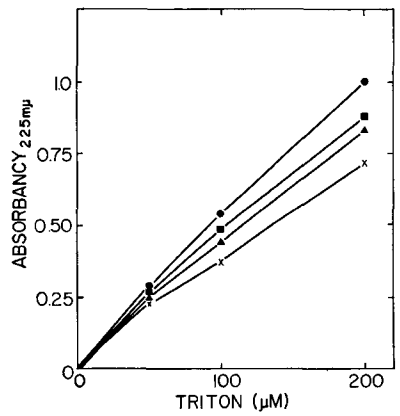

FIGURE 9 Adsorption of Triton to chloroplast membranes. Relative Triton concentrations were determined by measuring absorbancy of ether extracts at 225 mμ as described in Methods. ● is standard curve, no chloroplasts present. ■, ▲, × shows disappearance of Triton from medium produced by chloroplast concentrations of respectively 10, 20, and 40 μg chlorophyll per ml.

effective DBS concentration for producing swelling was 100–200 μM, similar to that found for Triton.

A micrograph of a chloroplast swollen in 104 μM DBS published by Itoh et al. (23) in their paper indicated that apparently vesicle formation had occurred. The main difference between DBS- and Triton-treated chloroplasts seemed to be that in the former no continuous enclosing membrane was present, and grana were not drawn out into long strands but were simply "bubbling." These workers did not suggest any mechanism for the swelling caused by DBS. However, they did propose that the first state in DBS lysis was to break the chloroplast down into swollen grana, since counts of particles obtained after lysis corresponded to estimates of grana number per chloroplast. This does not seem to be true of Triton-treated chloroplasts, electron micrographs of which clearly show the loss of all grana structure during swelling and the production of presumably spherical vesicles from the lamellar membranes.

Triton seems to offer a very mild method of unfolding chloroplast structure for ultrastructural investigations, and is an alternative to the techniques of osmotic shock and sonication which have been employed in the past. Three observations have arisen from present studies.

(*a*) Closed loop formations are apparent at the end of many of the darker membranes which seemingly are drawn-out membranes of grana disks (Fig. 8). In untreated chloroplasts these darker membranes are interpreted as being points at which the unit membranes of grana disks are in contact. These unit membranes are usually tightly held together, but Triton seems to "split" them into two single membranes. In the remarkable structures demonstrated by Izawa and Good (24)

TABLE I

Adsorption of Triton ×-100 to Chloroplast Membranes

Triton	Chloro-phyll	Triton adsorbed chlorophyll	Average	Area of Triton (T)	Area of Chloroplasts (C)	Ratio T/C
μM	μg/ml	μmoles/mg		μ^2/mg chl	μ^2/mg chl	
50	10	0.3				
50	20	0.4	0.3	0.8×10^{11}	8×10^{11}	0.1
50	40	0.3				
100	10	1.1				
100	20	0.9	0.9	2.2×10^{11}	8×10^{11}	0.3
100	40	0.75				
200	10	2.4				
200	20	1.8	1.9	4.5×10^{11}	8×10^{11}	0.5
200	40	1.4				

Varying amounts of chloroplasts were added to 10 ml of 50, 100, and 200 μM Triton solutions in 0.1 M NaAc, pH 6.0, and centrifuged 5 min later at 10,000 *g*. Triton uptake was measured by its disappearance from the medium as described in Methods.

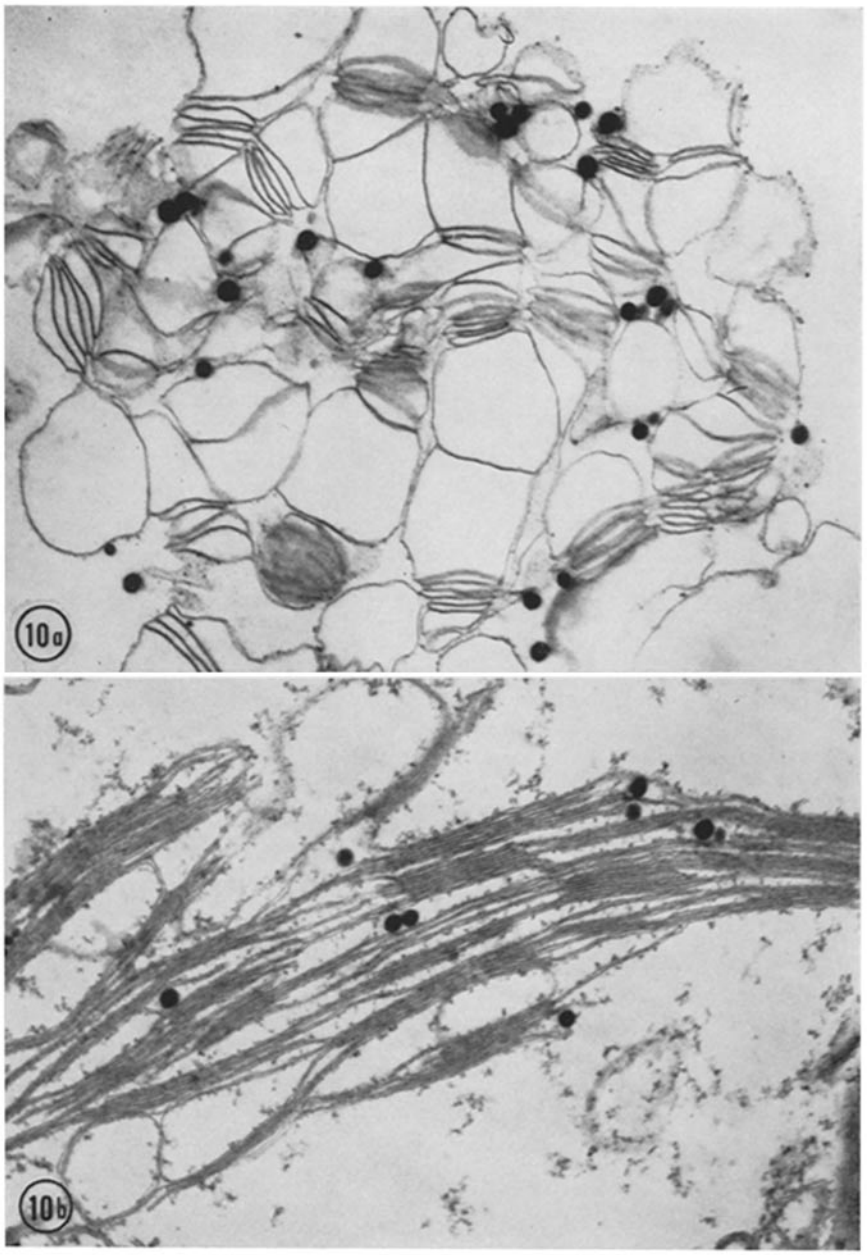

FIGURE 10 (*a*) Chloroplast suspended in 0.05 M sodium acetate, pH 6.0, and 5% dextran. Some swelling has occurred in this instance, due to the low osmotic strength of the medium. × 20,000. (*b*) Chloroplast from the same preparation after Triton addition to 100 μM. In the presence of dextran and other high molecular weight materials, shrinkage occurs when Triton is added. × 20,000.

in EDTA-treated chloroplasts suspended in solutions of low osmotic strength, all of the grana membranes seem to be split in a similar manner and illumination in the presence of atebrin causes reformation of the dual membrane structure.

(*b*) "Osmiophilic granules" become more numerous in treated chloroplasts and show greater variation in size (Figs. 7, 8). These probably do not represent the original population of normal osmiophilic granules; the larger granules may be

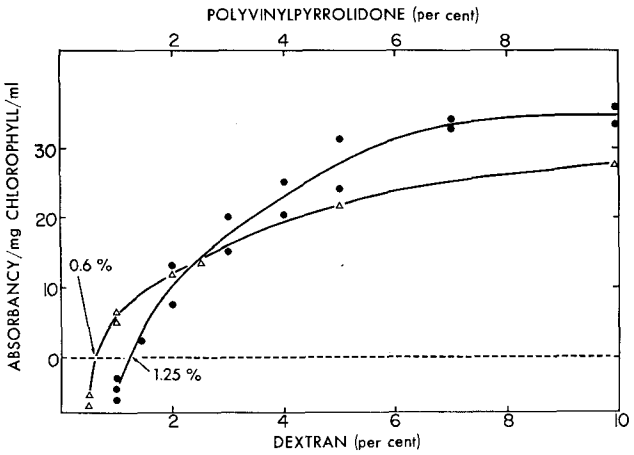

FIGURE 11 Shrinkage of chloroplasts after addition of Triton to a final concentration of 100 μM. Chloroplasts were suspended in varying concentrations of nonpenetrating, osmotically active materials in 0.1 M NaCl, pH 6.0. ● shows dextran; △ is polyvinylpyrrolidone.

composed of normal granules which have coalesced, and smaller granules are possibly a breakdown of organized membrane lipid structure into a less organized state. It is interesting that most of the larger granules are surrounded by a circular membrane (Fig. 8). There has been some question as to whether osmiophilic granules are membrane bounded (25, 26). At least after Triton treatment they seem to be within a membranous enclosure of some sort.

(*c*) Unless a drastic reformation of membranes has occurred following Triton treatment, it appears that even in Class II chloroplasts there is a continuous membrane enclosing all of the internal structures, as can be clearly seen in Fig. 8. This membrane may be much enfolded in untreated chloroplasts but swells to a balloon-like structure in 200 μM Triton. In 100 μM Triton (Fig. 7) it can be seen in the formative stage and osmiophilic granules seem to adhere to it. It is obvious that if this membrane breaks, the next layer of membranes could easily form a new enclosure.

Mechanism of Triton Action and Relation to Chloroplast Permeability

Neumann and Jagendorf (6) found that under conditions of pH and concentration where Triton acts as an uncoupler chloroplasts increased in volume by approximately four times, a somewhat larger increment than determined by our methods. These workers also suggested that the major effect of Triton was a production of pores, followed by ionic equilibration, which our results fully support. Pores produced in the chloroplast membranes by detergents would satisfactorily explain the inhibition of functional parameters reported here. Any hydrogen ions transported into the chloroplast during illumination would immediately leak back out and no pH gradient could be maintained. Therefore, all light-induced conformational changes would also be inhibited by detergents, since these changes have been shown to be primarily dependent on hydrogen ion transport (12).[2] Since photophosphorylation seems to be intimately linked to hydrogen ion transport (6, 7), it would also be drastically reduced. Finally, it would be expected that, if Triton was producing a single basic alteration of membrane structure from which its other inhibitory actions were derived, the inhibitory concentrations should be similar, and it was found that 60–120 μM Triton did in fact completely inhibit all of the functional parameters measured.

Neumann and Jagendorf further indicated that water movement into the chloroplast was controlled by an influx of ions through the pores produced by Triton, with consequent swelling. However, our results suggest that, although an initial ionic movement into the chloroplast could contribute to swelling under some circumstances, the main water movement is controlled by a fixed, osmotically active material within the chloroplast.

This argument is strongly supported by the experiments in which Triton is added to chloroplasts suspended in salt solutions in the presence of dextran, polyvinylpyrrolidone or bovine serum albumin. Under these conditions a striking shrinkage, rather than swelling, occurs. The following mechanism is proposed to account for this finding.

Normally when Triton is added to chloroplast

suspensions the chloroplast membranes become completely permeable to small ions, probably because uncharged "pores" are produced at the binding sites of Triton. These pores are not necessarily true holes in the membrane, but probably represent points at which adsorbed Triton molecules have displaced normal membrane constituents. Ions flow across the chloroplast membranes until equilibrium is attained and water then enters in response to fixed, osmotically active species within, so that the chloroplasts swell. It is therefore inconsequential whether the initial movement of ions is inward or outward; as soon as ionic equilibrium is attained (which occurs very rapidly) water begins to flow inward. However, if the osmolarity of the nonpenetrating osmotically active species outside (dextran, PVP, etc.) is greater than the osmolarity of the fixed species within the chloroplasts, then addition of Triton allows equilibration of small ions followed by movement of water *outward* into the medium which is now hypertonic with respect to the inside.

Some time ago Jacobs and Stewart (5) proposed a similar explanation for the behavior of erythrocytes when they are exposed to surface active materials in the presence of sucrose. Very small quantities of sucrose protect red cells against the hemolysis which typically occurs when they are made permeable to cations. The distribution of water in red cells is normally a function of the relative concentrations of internal and external osmotically active species, as determined by the equilibrium of penetrating anions. When surface active agents are added, cations can also equilibrate and the internal water is then determined by the equation $w = \frac{P}{C_m}$, where w is the ratio of the new and original cell water volumes, and P and C_m are respectively the original internal and external concentrations of nonpenetrating molecules. The amount of water in the cell is then controlled solely by the external concentration of non-penetrating molecules, since the amount of internal nonpenetrating osmotically active species is constant. For instance, if the external osmolarity of sucrose, a nonpenetrating neutral molecule, is equivalent to the internal osmolarity of hemoglobin, the erythrocytes will not swell and hemolyse when they are made permeable to small ions. The same argument may be applied to chloroplasts, although the material composing the fixed, osmotically active species within the chloroplast is still unknown. In this case it is the external concentration of dextran or PVP which controls water movement across the membrane after ions have attained equilibrium. The external concentration of these materials at which neither swelling nor shrinking occurs in 0.1 M salt solution should therefore be equal to the internal concentration of the fixed, osmotically active material at the particular volume of chloroplasts under these conditions. This value is 0.05 milliosmol for dextran and 0.15 milliosmol for PVP. The average, 0.1 milliosmol, is probably a reasonable estimate of the internal concentration of the fixed species.

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